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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/00, C07K 14/435

A1

(11) International Publication Number: WO 96/23898

(43) International Publication Date: 8 August 1996 (08.08.96)

(21) International Application Number: PCT/DK96/00052

(22) International Filing Date: 31 January 1996 (31.01.96)

(30) Priority Data:

| | | |
|---|---|---|
| 0110/95 | 31 January 1995 (31.01.95) | DK |
| 0982/95 | 7 September 1995 (07.09.95) | DK |

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

(54) Title: A METHOD OF DETECTING BIOLOGICALLY ACTIVE SUBSTANCES

(57) Abstract

The present invention relates to a method of detecting a biologically active substance affecting intracellular processes, the method comprising: (a) culturing a cell containing a DNA sequence coding for (i) a green fluorescent protein (GFP) having a protein kinase recognition site, or (ii) a green fluorescent protein wherein one or more amino acids have been substituted, inserted or deleted to provide a binding domain of a second messenger or an enzyme recognition site, or (iii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GFP and an enzyme recognition site or a binding domain for a second messenger under conditions permitting expression of the DNA sequence; (b) measuring the fluorescence of the cell; (c) incubating the cell with a sample suspected of containing a biologically active substance affecting intracellular processes; and (d) measuring the fluorescence produced by the incubated cell and determining any change in the fluorescence compared to the fluorescence measured in step (b), such change being indicative of the presence of a biologically active substance in said sample.

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# A METHOD OF DETECTING BIOLOGICALLY ACTIVE SUBSTANCES

## FIELD OF INVENTION

The present invention relates to a method of detecting biologically active substances affecting intracellular processes, and a DNA construct and a cell for use in the method.

## BACKGROUND OF THE INVENTION

Second messengers and protein kinases play key roles in the signalling pathways that control the response of mammalian cells (and probably all eukaryotic cells) to most stimuli. Although such signalling pathways have been subjected to extensive studies, detailed knowledge on e.g. the exact timing and spatial characteristics of signalling events is often difficult to obtain due to lack of a convenient technology. There is, however, one exception to this rule: our understanding of the role of $Ca^{2+}$ in e.g. intracellular signalling has been greatly improved due to the development of the fluorescent $Ca^{2+}$ probe FURA-2 that permits real times studies of $Ca^{2+}$ in single living cells.

Moreover, the construction of probes for cAMP (Adams et al., **Nature 349** (1991), 694-697) and activity of the cAMP-dependent protein kinase (Sala-Newby and Campbell, **FEBS 307(2)** (1992), 241-244) has been attempted. The protein kinase A probe, however, suffers from the drawback that it is based on the firefly luciferase and accordingly produces too little light for fast single cell measurements. The cAMP probe on the other hand has to be microinjected and is therefore not well suited for routine laboratory work. In conclusion, both probes lack some of the elegant properties that resulted in the widespread use of FURA-2.

Recently it was discovered that Green Fluorescent Protein (GFP) expressed in many different cell types, including mammalian cells, became highly fluorescent (Chalfie et al., **Science 263** (1994), 802-805). WO/07463 describes a cell capable of expressing GFP

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and a method for selecting cells expressing a protein of interest and GFP basec detection of GFP-fluorescence in the cells.

## SUMMARY OF THE INVENTION

The purpose of the present invention is to provide a method of detecting a biologi active substance affecting intracellular processes based on the use of green fluores protein, including wild-type GFP derived from the jelly fish *Aequorea victoria* modifications of GFP, such as modifications that changes the spectral properties of GFP fluorescence, for the construction of probes, preferably real time probes second messengers and protein kinase activity.

In one aspect, the present invention relates to a DNA construct comprising a D sequence coding for

(i) green fluorescent protein (GFP) wherein one or more amino acids have b substituted, inserted or deleted to provide a binding domain of a second messe or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified ( and a binding domain of a second messenger or an enzyme recognition site.

In another aspect, the present invention relates to a cell containing a DNA sequ coding for

(i) green fluorescent protein wherein one or more amino acids have l substituted, inserted or deleted to provide a binding domain of a second messe or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GFP and a binding domain of a second messenger or an enzyme recognition site,

and capable of expressing said DNA sequence.

In a further aspect, the present invention relates to a method of detecting a biologically active substance affecting intracellular processes, the method comprising

(a) culturing a cell containing a DNA sequence coding for

(i) green fluorescent protein wherein one or more amino acids have been substituted, inserted or deleted to provide a binding domain of a second messenger or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GFP and a binding domain of a second messenger or an enzyme recognition site

under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample suspected of containing a biologically active substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining any change in the fluorescence compared to the fluorescence measured in step (b), such change being indicative of the presence of a biologically active substance in said sample.

In a still further aspect, the present invention relates to a method of characterizing the biological activity of a substance with biological activity, the method comprising

(a) culturing a cell containing a DNA sequence coding for

(i) green fluorescent protein wherein one or more amino acids have be substituted, inserted or deleted to provide a binding domain of a second messen; or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified G and a binding domain of a second messenger or an enzyme recognition site

under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample of a biologically active substance affect intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining a change in the fluorescence compared to the fluorescence measured in step (b), s change being characteristic of the biological activity of the biologically active substai in said sample.

Furthermore, studies on the substrate specificity of the different protein kinase (PKA) isoforms using synthetic peptides have shown that peptides containing the mo **RRXSX** or **RXKRXXSX** (S being the phosphorylated amino acid) tend to be the b substrates for PKA, and a review by Zetterquist, Ö. et al. (in Kemp, B.E. (ed.) **Pept and Protein Phosphorylation** (1990), 172-188, CRC Press, Boca Raton, Florida, US confirms that most known substrates of PKA contain said motifs.

Available amino acid sequences of GFP do not suggest that GFP is a PKA substr because of a lack of recognition sites comprising the motifs **RRXSX** or **RXKRXX** It is therefore surprising that a native or wild-type green fluorescent protein (GI derived from the jellyfish *Aequorea victoria* can be phosphorylated by protein kinas

and thereby the spectral properties of GFP are changed resulting in a substantial increase of fluorescence.

In a preferred aspect, the present invention relates to a method of detecting a biologically active substance affecting intracellular processes, the method comprising

(a) culturing a cell containing a DNA sequence coding for a wild-type green fluorescent protein having a protein kinase recognition site under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample suspected of containing a biologically active substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining any change in the fluorescence compared to the fluorescence measured in step (b), such change being indicative of the presence of a biologically active substance in said sample.

In a further preferred aspect, the present invention relates to a method of characterizing the biological activity of a substance with biological activity, the method comprising

(a) culturing a cell containing a DNA sequence coding for a wild-type green fluorescent protein having a protein kinase recognition site, under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample of a biologically active substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining a: change in the fluorescence compared to the fluorescence measured in step (b), sa change being characteristic of the biological activity of the biologically active substan in said sample.

In a still further preferred aspect the present invention relates to a DNA constru comprising the DNA sequence shown in Fig. 4a coding for a wild-type GFP and transformed cell containing said DNA construct and capable of expressing said DN sequence. The transformed cell is preferably a mammalian cell. A microorganism, coli NN049087, carrying the DNA sequence shown in Fig. 4a has been deposited f the purpose of patent procedure according to the Budapest Treaty in Deutscl Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1 b, I 38124 Braunschweig, Federal Republic of Germany, under the deposition No. DS 10260.

In the present context, the term "green fluorescent protein" is intended to indicate protein which, when expressed by a cell, emits fluorescence (cf. Chalfie et al., Scien 263, 1994, pp. 802-805). In the following, GFP in which one or more amino acids ha been substituted, inserted or deleted is most often termed "modified GFP".

The term "second messenger" is used to indicate a low molecular weight compone involved in the early events of intracellular signal transduction pathways.

The term "binding domain of a second messenger" is used to indicate a segment of protein which, in the course of intracellular metabolic processes, binds the seconda messenger.

The term "enzyme recognition site" is intended to indicate a peptide sequen covalently modified by an enzyme (e.g. phosphorylated, glycosylated or cleave preferably the enzyme recognition site is a protein kinase recognition site, which intended to indicate a peptide sequence covalently modified by a kinase, i phosphorylated.

It should be emphasized that phosphorylation of a protein at a protein kinase recognition site often is followed (or accompanied) by dephosphorylation of said protein. A GFP based probe for activity of given protein kinase(s) would therefore also provide information on the activity of relevant protein phosphatases since the parameter monitored is the net phosphorylation of the GFP based probe.

The term "hybrid polypeptide" is intended to indicate a polypeptide which is a fusion of at least a portion of each of two proteins, in this case at least a portion of the green fluorescent protein and at least a portion of a binding domain of a second messenger or an enzyme recognition site.

In the present context, the term "biologically active substance" is intended to indicate a substance which has a biological function or exerts a biological effect in the human or animal body. The sample may be a sample of a biological material such as a microbial extract, or it may be a sample containing a compound or mixture of compounds prepared by organic synthesis.

The phrase "any change in fluorescence" means any change in absorption properties, such as wavelength and intensity, or any change in spectral properties of the emitted light, such as a change of wavelength, fluorescence lifetime, intensity or polarisation.

The mechanism(s) behind a change in e.g. the fluorescence intensity of a modified GFP upon phosphorylation could be several. As one possibility, phosphorylation of said GFP variant could change the chromophore environment, either due to proximity of the added phosphate group or to phosphorylation induced conformational changes. Correspondingly, binding of e.g. a second messenger to the binding domain of a some GFP variant or GFP fusion protein could induce conformational changes that ultimately changes the chromophore environment and thereby the fluorescence. As support for these suggestions, it has been shown that amino acid substitutions distant to the chromophore (e.g. amino acids 167, 202, 203 and 222) can change the fluorescence intensity and

spectral characteristics of GFP (Ehrig et al. (1995) FEBS Letters 367:163; Heim et al. (1994) Proc. Natl. Acad. Sci. 91:12501).

The development of luminescent probes according to the present invention allows re time studies of second messengers and specific enzymes such as protein kinases single living cells, thereby making it possible to study the precise timing and the spat: characteristics of these factors. Moreover, studies on heterogeneity in cell populatio are made possible.

Due to the strong fluorescence of GFP, the luminescence of cells expressing the prob may easily be detected and analyzed by employing a combination of fluorescen microscopy and image analysis. Furthermore, it should be emphasized that the prob described are easy to introduce into cells, as they can be expressed in the cells interest after transfection with a suitable expression vector.

Real time recombinant probes for second messengers and enzyme activity, such kinase activity, are not only useful in basic research but also in screening programm aiming at identifying novel biologically active substances. Many currently used screeni programmes designed to find compounds that affect cAMP concentration and prot kinase activity are based on receptor binding and/or reporter gene expression. T recombinant probes described herein, on the other hand, make it possible to devel an entirely new type of screening assays able to monitor immediate and transi changes of cAMP concentration and protein kinase activity in intact living cells.

Any novel feature or combination of features described herein is considered essen to this invention.

## DETAILED DESCRIPTION OF THE INVENTION

In a preferred embodiment of the present invention, the gene encoding GFP is deri from the jellyfish *Aequorea victoria*. The sequence of this gene is described in Pras et al., Gene 111, 1992, pp. 229-233 (GenBank Accession No. M62653). The gene r

be modified so as to code for a variant GFP in which one or more amino acid residues have been substituted, inserted or deleted to provide a binding domain of a second messenger or an enzyme recognition site. According to this embodiment, it is preferred to insert a DNA sequence coding for an enzyme recognition site into the gene coding for GFP, for instance at one of the following positions: between amino acid 39 and 40, between amino acid 71 and 72, between amino acid 79 and 80, between amino acid 107 and 108, between amino acid 129 and 130, between amino acid 164 and 165, or between amino acid 214 and 215. Points of insertion may be selected on the basis of surface probability (which may be calculated using the GCG software package which employs a formula of Emini et al., J. Virol. 55(3), 1985, pp. 836-839). When the enzyme is protein kinase C, the recognition site inserted should preferably contain the motif XRXXSXRX, S being the phosphorylated amino acid. In successful constructs of this type, phosphorylation of the modified GFP may result in detectably altered optical proterties of GFP. It should be noted that extensive deletion may result in loss of the fluorescent properties of GFP. It has been shown, that only one residue can be sacrificed from the amino terminus and less than 10 or 15 from the carboxyl terminus before fluorescence is lost, cf. Cubitt et al. TIBS Vol. 20 (11), pp. 448-456, November 1995. Thus, according to this invention the modification of the GFP gene so as to code for a variant GFP in which one or more amino acid residues have been substituted, inserted or deleted is limited to modifications resulting in a variant GFP having fluorescence properties.

The binding domain of a second messenger may be a receptor of a second messenger. The second messenger may be cyclic AMP, inositol phosphate 3, cyclic GMP, cyclic ADP or diacylglycerol. The binding domain is preferably the cyclic AMP receptor (CRP, e.g. as described in Weber and Steitz, J. Mol. Biol. 198, 1987, pp. 311-326; Schroeder and Dobrogosz, J. Bacteriol. 167, 1986, pp. 612-622) or a part thereof capable of binding cyclic AMP.

Native CRP has two distinct domains: an N-terminal cAMP binding domain as well as a C-terminal DNA binding activity (Weber and Steitz, J. Mol. Biol. 198 (1987), 311-326). Upon binding of cAMP to the N-terminal portion of CRP a conformational

change is induced in the C-terminus, which allows the binding of CRP to the promote of certain genes. In the successful fusions of CRP (or a portion thereof) to GFP (or portion thereof), cAMP induced conformotional changes in CRP are transmitted GFP, thereby changing the optical properties of GFP.

In a preferred embodiment of the present invention, the gene or cDNA sequen encoding a wild-type GFP is derived from the jellyfish *Aequorea victoria*. A preferr sequence of this gene is disclosed by Fig. 4a herein. Fig. 4a shows the nucleoti sequence of a wild-type GFP (Hind3-EcoR1 fragment) and Fig. 4b shows the ami acid sequence, wherein start codon ATG corresponds to position 8 and stop cod TAA corresponds to position 722 in the nucleotide sequence of Fig. 4a. Anoth sequence of an isotype of this gene is disclosed by Prasher et al., Gene 111, 1992, p 229-233 (GenBank Accession No. M62653). Any gene that codes for a fluoresce protein, such as wild-type GFP, having a protein kinase recognition site, and deriv from any organism expressing a green fluorescent protein or similar fluoresce phosphorescent or luminescent protein may be used in this invention.

The enzyme recognition site or protein kinase recognition site is preferably a Ser/T or Tyr protein kinase, such as protein kinase C or a protein kinase A recognition s (both are reviewed in e.g. B.E. Kemp and R.B. Pearson, TIBS 15, Sept. 1990, pp. 3 346), or the insulin receptor or the Src kinase or a portion thereof containing a mo required as a substrate for protein kinase, as suggested above. Kinase catalys phosphorylation may result in detectably altered optical properties of GFP.

The DNA sequence encoding GFP, the binding domain of a second messenger or t enzyme recognition site may suitably be of genomic or cDNA origin, for instan obtained by preparing a suitable genomic or cDNA library and screening for DN sequences coding for all or part of any of these proteins by hybridization using synthe oligonucleotide probes in accordance with standard techniques (cf. Sambrook et supra).

The DNA construct of the invention encoding the wild-type GFP, modified GFP or hybrid polypeptide may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors. For most purposes, it may be practical to prepare a shorter DNA sequence such as the DNA sequence coding for the enzyme recognition site synthetically, while the DNA coding for GFP or the binding domain of a second messenger will typically be isolated by screening of a DNA library.

Furthermore, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA construct, in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory, New York, USA).

The DNA construct may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or Saiki et al., Science 239 (1988), 487 - 491. A more recent review of PCR methods may be found in PCR Protocols, 1990, Academic Press, San Diego, California, USA.

The DNA sequence coding for GFP may also be modified by other means such as by conventional chemical mutagenesis or by insertion, deletion or substitution of one or more nucleotides in the sequence, either as random or site-directed mutagenesis. It is expected that such mutants will exhibit altered optical properties or altered heat stability.

The DNA construct of the invention may be inserted into a recombinant vector which may be any vector which may conveniently be subjected to recombinant DNA procedures. The choice of vector will often depend on the host cell into which it is to

be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vec which exists as an extrachromosomal entity, the replication of which is independent chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one whi when introduced into a host cell, is integrated into the host cell genome and replicat together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encodi wild-type GFP, the modified GFP or the hybrid polypeptide is operably linked additional segments required for transcription of the DNA. In general, the expressi vector is derived from plasmid or viral DNA, or may contain elements of both. T term, "operably linked" indicates that the segments are arranged so that they functi in concert for their intended purposes, e.g. transcription initiates in a promoter a proceeds through the DNA sequence coding for the modified GFP or hyb polypeptide

The promoter may be any DNA sequence which shows transcriptional activity in t host cell of choice and may be derived from genes encoding proteins either homologc or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequen encoding wild-type GFP, the modified GFP or hybrid polypeptide in mammalian ce are the SV40 promoter (Subramani et al., Mol. Cell Biol. 1 (1981), 854 -864), the M7 (metallothionein gene) promoter (Palmiter et al., Science 222 (1983), 809 - 814) or t adenovirus 2 major late promoter.

An example of a suitable promoter for use in insect cells is the polyhedrin promot (US 4,745,051; Vasuvedan et al., FEBS Lett. 311, (1992) 7 - 11), the P10 promot (J.M. Vlak et al., J. Gen. Virology 69, 1988, pp. 765-776), the *Autographa californi* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immedia early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delaye early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255 (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1 (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304 (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gen, or the *Bacillus pumilus* xylosidase gene, or by the phage Lambda $P_R$ or $P_L$ promoters or the E. coli lac, trp or tac promoters.

The DNA sequence encoding wild-type GFP, the modified GFP or hybrid polypeptide of the invention may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant vector may further comprise a DNA sequence enabling the vector replicate in the host cell in question. An example of such a sequence (when the h cell is a mammalian cell) is the SV40 origin of replication.

When the host cell is a yeast cell, suitable sequences enabling the vector to replic are the yeast plasmid 2$\mu$ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of whi complements a defect in the host cell, such as the gene coding for dihydrofola reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin or hygromycin. F filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, sC.

The procedures used to ligate the DNA sequences coding for wild-type GFP, t modified GFP or hybrid polypeptide, the promoter and optionally the terminator and/ secretory signal sequence, respectively, and to insert them into suitable vectc containing the information necessary for replication, are well known to persons skill in the art (cf., for instance, Sambrook et al., op.cit.).

The host cell into which the DNA construct or the recombinant vector of the inventic is introduced may be any cell which is capable of expressing the present DNA constru and includes bacteria, yeast, fungi and higher eukaryotic cells, such as mammalian cell

Examples of bacterial host cells which, on cultivation, are capable of expressing tl DNA construct of the invention are grampositive bacteria such as strains of *Bacillu* such as strains of *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilu B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megatheriu* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, gramnegative bacteria such as *Echerichia coli*. The transformation of the bacteria m be effected by protoplast transformation or by using competent cells in a mann known per se (cf. Sambrook et al., supra).

Examples of suitable mammalian cell lines are the HEK293 and the HeLa cell lines, primary cells, and the COS (e.g. ATCC CRL 1650), BHK (e.g. ATCC CRL 1632, ATCC CCL 10), CHL (e.g. ATCC CCL39) or CHO (e.g. ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845.

Examples of suitable yeasts cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are described, e.g. in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the modified GFP or hybrid polypeptide may be preceded by a signal sequence and optionally a leader sequence , e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279).

Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 230 023, EP 184 438.

When a filamentous fungus is used as the host cell, it may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally con-

sidered to be an advantage as the DNA sequence is more likely to be stably maintain in the cell. Integration of the DNA constructs into the host chromosome may be pe formed according to conventional methods, e.g. by homologous or heterologo recombination.

Transformation of insect cells and production of heterologous polypeptides therein m be performed as described in US 4,745,051; US 4,879,236; US 5,155,037; 5,162,222; E 397,485) all of which are incorporated herein by reference. The insect cell line used the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as describe in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementione references.

The transformed or transfected host cell described above is then cultured in a suitab nutrient medium under conditions permitting the expression of the present DN construct after which the cells may be used in the screening method of the inventio Alternatively, the cells may be disrupted after which cell extracts and/or supernatan may be analysed for fluorescence.

The medium used to culture the cells may be any conventional medium suitable fo growing the host cells, such as minimal or complex media containing appropria supplements. Suitable media are available from commercial suppliers or may b prepared according to published recipes (e.g. in catalogues of the American Typ Culture Collection).

In the method of the invention, the fluorescence of cells transformed or transfecte with the DNA construct of the invention may suitably be measured in a spectromete where the spectral properties of the cells in liquid culture may be determined as scar of light excitation and emission. Alternatively, such cells grown on nitrocellulose filte placed on plates containing solid media may be illuminated with a scannin polychromatic light source and imaged with an integrating colour camera. The colo

of the emitted light may then be determined by image analysis using specialised software.

The invention is further illustrated in the following examples with reference to the appended drawings, wherein

Fig. 1 shows a map of the pUC19-GFP plasmid construction. GFP nucleotide numbers referred to below with a "G" are from the GenBank GFP sequence record (accession No. M62653). Bases in italics represent GFP sequence. The pUC19 nucleotide numbers referred to below with a "P" are from the GenBank pUC19 sequence record (accession No. X02514). Bases in plain text represent pUC19 sequence. Bases in bold represent non-GFP non-pUC19 sequence, which have been inserted by PCR for the introduction convenient restriction sites.

Fig. 2 shows maps of the four basic GFP-CRP fusion constructs:
A) Full length GFP at the N-terminal fused with full length CRP at the C-terminal.
B) Truncated GFP at the N-terminal fused with full length CRP at the C-terminal.
C) Full length CRP at the N-terminal fused with full length GFP at the C-terminal.
D) Truncated CRP at the N-terminal fused with full length GFP at the C-terminal, corresponding to the construct where the DNA binding domain of CRP has been replaced with GFP.

GFP nucleotide numbers referred to below with a "G" are from the GenBank GFP sequence record (accession No. M62653). CRP nucleotide numbers referred to below with a "C" are from the GenBank CRP sequence record (accession No. M13770). The pUC19 nucleotide numbers referred to below with a "P" are from the GenBank pUC19 sequence record (accession No. X02514).

Example 1

Cloning of cDNA encoding the green fluorescent protein

Briefly, total RNA, isolated from *A. victoria* by a standard procedure (Sambrook et Molecular Cloning. 2., eds. (1989) (Cold Spring Harbor Laboratory Press: Cold Spr Harbor, New York), 7.19-7.22) was converted into cDNA by using the AMV reve transcriptase (Promega, Madison, WI, USA) as recommended by the manufactu The cDNA was then PCR amplified, using PCR primers designed on the basis c previously published GFP sequence (Prasher et al., Gene 111 (1992), 229-2 GenBank accession No. M62653) together with the UlTma™ polymerase (Per Elmer, Foster City, CA, USA). The sequences of the primers were: GF TGGAATAAGCTTTATGAGTAAAGGAGAAGAACTTTT and GFP AAGAATTCGGATCCCTTTAGTGTCAATTGGAAGTCT

Restriction endonuclease sites inserted in the 5' (a HindIII site) and 3' (EcoRI a BamHI sites) primers facilitated the cloning of the PCR amplified GFP cDNA int slightly modified pUC19 vector. The details of the construction are as follows: La Shine-Dalgarno AGGA, immediately followed by the 5' HindIII site plus an extra T a the GFP ATG codon, giving the following DNA sequence at the lacZ-promoter G fusion point: $P_{LacZ}$-AGGAAAGCTTTATG-*GFP*. At the 3' end of the GFP cDNA, base pair corresponding to nucleotide 770 in the published GFP sequence (GenBa accession No. M62653) was fused to the EcoRI site of the pUC19 multiple cloning s (MCS) through a PCR generated BamHI, EcoRI linker region.

## Example 2

### Isolation of mutant GFPs

A variant of GFP with altered optical properties and/or heat stability is prepared subjecting the GFP described in Example 1 to a round of chemical mutagene followed by screening potential mutants for altered properties.

In brief, the GFP-encoding DNA sequence described in Example 1 (the HindIII-Eco fragment) is heat-denatured and subjected to chemical mutagens essentially described by Myer et al., Science 229, 1985, p. 242. The mutagen is either nitrous ac or permanganate or formic acid. The resulting mutated population of single stranc

GFP fragments are either amplified by PCR using the primers described in Example 1, or reverse transcribed by AMV reverse transcriptase as described in Example 1 prior to amplification by PCR. The PCR products are cleaved by restriction enzymes HindIII and EcoRI and the products of this reaction are ligated into the modified pUC19 plasmid described in Example 1.

The ligation reaction is transformed into an E. coli strain and plated on LB agar plates containing 100 μg/ml ampicillin to give approximately 500 transformants per plate. The fluorescence of GFP in the cells is detected by exciting the plates with a light source at 398 nm or 365 nm. Replicas of colonies are made onto fresh plates or plates on which a nitrocellulose filter has been placed prior to replication. When colonies have formed once more, they are individually collected and resuspended in water. The cell suspensions are placed in a LS50B Luminescence Spectrometer (Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England) equipped with a temperature-controlled cuvet holder, and the spectral properties (scans of both light excitation and emission) are determined. Alternatively, whole plates with approximately 500 transformants are illuminated with a scanning polychromatic light source (fast monochromator from T.I.L.L. Photonics, Munich, Germany) and imaged with an integrating RGB colour camera (Photonic Science Color Cool View). The actual colour of the emitted light was determined by image analysis using the Spec R4 software (Signal Analytics Corporation, Vienna, VA, USA).

Heat sensitivity of the mutated GFP is tested by characterizing its spectral properties, as described above, after a sequential rise of the temperature from 20°C to 80°C.

In another round of mutagenesis, *E.coli* cells containing the GFP pUC19 plasmid described in Example 1, are subjected to treatment with N-methyl-N-nitro-N-nitrosoguanidine at a concentration of 25 milligrams per liter for 18 hours, and the cells are plated and analyzed as described above. Alternatively, plasmids are first recovered from the treated cells and transformed into E.coli and plated and analyzed as described above.

Example 3

Construction of a GFP-based recombinant cAMP probe

The basis of the GFP-based recombinant cAMP probe described herein is the fusi of a portion of the cAMP receptor protein (CRP) from *E. coli* to GFP.

It was decided to prepare 4 basic GFP-CRP fusion constructs, from which a whc array of semi-random fusion constructs may be generated, some of which are expect to have the ability to induce conformational changes in GFP when cAMP is bound the N-terminal portion of CRP resulting in detectable changes in the optical properti of GFP.

*1. Description of the four basic GFP-CRP fusions*

The plasmid harbouring the GFP-CRP fusion shown in figure 2 A) was constructed t following way: The CRP insert of plasmid pHA7 (Aiba et al., Nucl. acids Res. 10 (198 1345-1377) was PCR amplified with the PCR primers
CRP1 (CGATACAGATCTAAGCTTTATGGTGCTTGGCAAACCGC) and
CRP-2 (CGGAATTCTTAAAAGCTTAGATCTTTACCGTGTGCGGAGATCA
followed by digestion with the restriction endonucleases BglII and EcoRI. The G insert of plasmid pUC19-GFP (see Example 1) was PCR amplified using the P primers GFP2 (see Example 1) and
GFP-4 (GAATCGTAGATCTTTGTATAGTTCATCCATGCCATG) followed digestion with the restriction endonucleases HindIII and BglII. Subsequently, in a thr part ligation, the BglII/EcoRI fragment of the PCR amplified CRP DNA and HindIII/BglII fragment of the PCR amplified GFP DNA was ligated with HindIII/EcoRI vector fragment of the slightly modified pUC19 plasmid described Example 1, followed by transformation of *E. coli*.

The plasmid harbouring the GFP-CRP fusion shown in figure 2 B) was constructed essentially as described above for the figure 2 A) plasmid with a single modification: The PCR primer GFP-3 (GAATCGTAGATCTTTGACTTCAGCACGTGTCTTGTA) was used instead of the GFP-4 PCR primer.

The plasmid harbouring the CRP-GFP fusion shown in figure 2 C) was made by PCR amplification of the CRP insert of plasmid pHA7 with PCR primers CRP1 and CRP-2, followed by digestion with restriction endonuclease HindIII and ligation into the HindIII site of plasmid pUC19-GFP (see Example 1).

The plasmid harbouring the GFP-CRP fusion shown in figure 2 D) was constructed essentially as described above for the figure 2 C) plasmid with a single modification: The PCR primer GFP-1 (CCAGTTAAGCTTAGATCTTCCGGGTGAGTCATAGCGTCTGG) was used instead of the CRP-2 PCR primer.

*2. Generation of semirandom GFP-CRP fusions*

The 4 basic GFP-CRP fusion plasmids described above are digested with the restriction endonuclease BglII (opening the plasmids at GFP-CRP fusion points), followed by treatment with the double stranded exonuclease Bal31 for 1 minute, 2 minutes, 3 minutes etc. up to 20 minutes (cf. Sambrook et al., op. cit. at 15.24). Subsequently, the Bal31 treated DNA is incubated with the T4 DNA polymerase (cf. Sambrook et al., op. cit. at 15.24) to generate blunt ends, followed by self ligation (essentially as described by Sambrook et al., op. cit. at 1.68). Finally, the self ligated Bal31 treated plasmid DNA is transformed into *E. coli.*

*3a. Screening of the CRP-GFP fusions for cAMP induced changes in fluorescence*

E. coli transformants expressing one of the four basic CRP-GFP fusions or one of the semirandom GFP-CRP fusions are grown overnight in 2 ml Luria-Bertani medium with

added ampicilin (100 $\mu$g/ml). The cells are then pelleted by centrifugation followed resuspension in 0.5 ml lysis buffer (100 mM NaCl, 1 mM EDTA and 50 mM Tris p 8.0). Subsequently, 25 $\mu$l 10 mg/ml Lysozyme is added to the resuspended ce followed by incubation for 10 min. at room temperature, vigorous vortexing a centrifugation for 5 min. at 20 000 x g. Finally, emission and excitation spectra for t resulting protein extracts (the supernatants) are acquired by using the LS5 Luminescence Spectrometer and the FL Data Manager software package (both fr Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England). The spectra record before as well as after the addition of cAMP to a final concentration of 0.5 mM a compared by using the Graph Builder software package (Perkin Elmer). The CRP-G fusions exhibiting cAMP induced changes in fluorescence are investigated further expression in mammalian cells.

*3b. (alternative protocol) Screening of the CRP-GFP fusions for cAMP induced chan in fluorescence.*

Cyclic AMP levels in E.coli cells vary according to the carbon source provided; see e Epstein et.al. (1975), Proc.Natl.Acad.Sci.USA 72, pp. 2300-2304, and Botsford a Harman (1992), Microbiological Reviews 56, p. 100-122. For example, cells grown glucose contain a lower level of cAMP than cells grown on e.g. glycerol. Furthermo shifting cells from one carbon source to another, or adding glucose to a culture gro on a non-glucose carbon source change the cAMP level of the cells. Hence, the cAM induced change in the fluorescence of the CRP-GFP fusions may be determined continuously measuring the fluorescence of cells expressing the fusions, after trans from medium containing e.g. glycerol as carbon to medium containing 0.2% gluc The cells are analyzed in liquid culture in the LS50B Luminescence Spectrometer by growing them on nitrocellulose filters placed on plates with solid media; the fil is transferred from plates with one type of medium to plates with another type medium, and the fluorescence is continuously monitored by exciting the plates wit scanning polychromatic light source (fast monochromator from T.I.L.L. Photon Munnich, Germany) and collecting colour images with an integrating RGB cc camera (Photonic Science Color Cool View). The actual colour of the emitted ligh

determined by image analysis using the Spec R4 software package (Signal Analytics Corporation, Vienna, VA, USA).

Example 4

Construction of a GFP based recombinant probe for protein kinase activity

*Description of the GFP based recombinant protein kinase C (PKC) substrates*

Studies on the substrate specificity of the different PKC isoforms using synthetic peptides have shown that peptides containing the motif XRXXSXRX (S being the phosphorylated amino acid) tend to be the best substrates for PKC (as reviewed in Kemp, B. E. and Pearson, R. B. (1990) **TIBS** 15 Sept., 342-346). Moreover, the naturally occurring neuronal PKC substrate GAP-43 has the following amino acid sequence around the phosphorylated serine residue (underlined): AATKIQASFRGHIT (Kosik, K.S et al. (1988) **Neuron 1,** 127-132). On the basis of these data we have selected the putative PKC recognition motif RQASFRS for insertion in GFP at various positions. Insertion points were selected on the basis of surface probability (calculated using the GCG software package, which employs a formula of Emini et al.(1985) **J. Virol., 55(3),** 836-839 ), slightly modified for the end values of the protein chains. The single probabilities are taken from Janin et al. (1978) **J. Mol. Biol. 125,** 357-386) and/or vicinity of the GFP chromophore. The heptapeptide is inserted in GFP by PCR at the following positions:

Between amino acid (aa) 39 and aa 40 (PCR primers PKC-1: GATACCAAAGATCTGAAAGAAGCTTGTCGGTATGTTGCATCACCTTCACC and PKC1: GATACCAAAGATCTGGAAAACTTACCCTTAAATTT),

between aa 52 and aa 53 (PCR primers PKC-2: GATACCAAAGATCTGAAAGAAGCTTGTCGTTTTCCAGTAGTGCAAATAAA and PKC2: GATACCAAAGATCTCTACCTGTTCCATGGCCAACAC),

between aa 71 and aa 72 (PCR primers PKC-3:

GATACCAAAGATCTGAAAGAAGCTTGTCGAAAGCATTGAACACCATAAG and PKC3: GATACCAAAGATCTTCAAGATACCCAGATCATATG), between aa 79 and 80 (PCR primers PKC-4: GATACCAAAGATCTGAAAGAAGCTTGTCGTTTCATATGATCTGGGTATCTar PKC4: GATACCAAAGATCTCAGCATGACTTTTTCAAGAGT), between aa 107 and 108 (PCR primers PKC-5: GATACCAAAGATCTGAAAGAAGCTTGTCGCTTGTAGTTCCCGTCATCTTT ar PKC5: GATACCAAAGATCTACACGTGCTGAAGTCAAGTTT), between aa 129 and 130 (PCR primers PKC-6: GATACCAAAGATCTGAAAGAAGCTTGTCGATCAATACCTTTTAACTCGATar PKC6: GATACCAAAGATCTTTTAAAGAAGATGGAAACATT), between aa 164 and 165 (PCR primers PKC-7: GATACCAAAGATCTGAAAGAAGCTTGTCGGTTAACTTTGATTCCATTCTT ar PKC7: GATACCAAAGATCTTTCAAAATTAGACACAACATT) and between aa 214 and 215 (PCR primers PKC-8: GATACCAAAGATCTGAAAGAAGCTTGTCGCTTTTCGTTGGGATCTTTCGAar PKC8: GATACCAAAGATCTAGAGACCACATGGTCCTTCTT).

The PCR primers were designed in the following way: Reverse primers: 5 GATACCAA AGA TCT GAA AGA AGC TTG TCG-3' + 21 nucleotides of th antisense strand (upstream of the second aa mentioned) and forward primers: 5 GATACCAA AGA TCT-3' + 21 nucleotides of the sense strand (downstream of th first aa), each PCR primer being provided with a unique BglII site (giving rise to th arginine and serine residues of the heptapeptide). The PKC site is inserted by PCR ( pUC19-GFP plasmid DNA (see Example 1) with the 8 forward primers and the matching reverse primers, followed by digestion with BglII, self-ligation an transformation of *E. coli* (cf. Sambrook et al., op. cit.).

*2. Screening of he GFP based recombinant PKC substrates for phosphorylation induce changes in fluorescence*

*E. coli* transformants expressing one of the eight GFP based recombinant PKC substrates are grown overnight in 2 ml Luria-Bertani medium with added ampicilin (100 $\mu$g/ml). The cells are then pelleted by centrifugation followed by resuspension in 0.5 ml lysis buffer (100 mM NaCl, 1 mM EDTA and 50 mM Tris pH 8.0). Subsequently, 25 $\mu$l 10 mg/ml Lysozyme is added to the resuspended cells, followed by incubation for 10 min. at room temperature, vigorous vortexing and centrifugation for 5 min. at 20 000 x g. Finally, emission and excitation spectra for the resulting protein extracts (the supernatants) are acquired by using the LS50B Luminescence Spectrometer and the FL Data Manager software package (Perkin Elmer). The spectra recorded before as well as after treatment of the extracts with purified PKC (Promega, Madison, WI, USA) according to the manufacturers instruction, are compared by using the Graph Builder software package (Perkin Elmer). The GFP based recombinant PKC substrates exhibiting phosphorylation induced changes in fluorescence are investigated further by expression in mammalian cells.

## Example 5

Characterization of the recombinant fusion probes in mammalian cells.

The CRP-GFP fusions (Example 3) exhibiting cAMP-induced changes in fluorescence as well as the GFP-based recombinant PKC substrates exhibiting phosphorylation-induced changes in fluorescence are investigated further by expression in mammalian cells.

Inserts of the respective plasmids are isolated by digestion with the restriction endonucleases HindIII and BamHI and ligated into the HindIII and BamHI sites of the MCS of the mammalian pREP4 vector (Invitrogen, San Diego, California, USA). Subsequently, Baby Hamster Kidney (BHK) are transfected with the resulting plasmid constructs according to the standard calcium phosphate-DNA precipitate protocol (cf. Sambrook et al., op. cit. at 16.33-16.35). Stable transfectants with high expression of the recombinant probes are identified and cloned after 6-14 days in culture by quantifying the fluorescence in an image analysis system, which consists of a Nikon Diaphot 200

microscope with a temperature controlled stage, a slow scan CCD camera (T.I.L Photonics), a polychromatic light source (T.I.L.L. Photonics), and a PC based ima analysis software package (FUCAL from T.I.L.L. Photonics). Alternatively, t fluorescence properties are monitored in a photometer based system. In this system t CCD camera is replaced by a photomultiplier D104 (PTI, Canada).

The clones are cultured for a couple of days in glass coverslip chambers (NUN Copenhagen, Denmark) before image analysis.

The ability of the clones to detect changes in cAMP is characterized by elevati intracellular cAMP level by challenging the cells with forskolin (0.1-10 $\mu$M) or dibutyr cAMP (1-100 $\mu$M) and monitoring the associated change of spectral properti Similarly, clones that are sensitive to variations in PKC activity are characterized activating PKC in them with PMA (phorbol 12-myristate 13-acetate) (10-1000 nM) OAG (1-oleoyl-2-acetyl-sn-glycerol) (1-100 $\mu$M). The stimulant-induced changes fluorescence properties are monitored continuesly using above mentioned imagi system. Combining imaging with photometry makes it possible to characterize t response of the recombinant probes in both high spatial and high temporal resolutic

## Example 6

## GFP as a recombinant probe for protein kinase activity

*Purification of GFP from E. coli cells expressing GFP*

*E. coli* cells containing a plasmid allowing expression of GFP were grown overnight 24°C. Cells were pelleted, the supernatant was discarded, and the pellet w resuspended in 1/20 of the original volume in 100mM Na-phosphate buffer (pH 8. Cells were disrupted by sonication, and cell debris were pelleted at 12000g for minutes. The supernatant was recovered, ammonium sulphate was added to a fi concentration of 1.5M, and the resulting solution was subjected to hydrophol interaction chromatography by applying it to a Phenyl-Sepharose CL-4B colur equilibrated with 1.5M ammunium sulphate. The column was eluted with water, a

fractions containing GFP were identified by green fluorescence when illuminated with 365nm UV light. To GFP containing fractions was added one volume of 20mM Tris, HCl (pH 7.5) and these were subjected to anion exchange chromatography by applying them to a Q-Sepharose column. The column was eluted with 20mM Tris, HCl (pH 7.5) + 1.0M NaCl. GFP containing fractions were identified by green fluorescence when illuminated with 365nm UV light. GFP containing fractions were subjected to gelfiltration by applying them to a Superose-12 column equilibrated with 100mM Na-phosphate buffer (pH 8.0). The column was eluted with 100mM Na-phosphate buffer (pH 8.0) and fractions containing GFP were identified by green fluorescence when illuminated with 365nm UV light. The resulting GFP preparation was greater than 95% pure as judged by HPLC analysis.

*In vitro GFP phosphorylation assay*

For *in vitro* phosphorylation of GFP, 0.5$\mu$g wild-type GFP (purified as described above) in 40mM Tris, pH 7.4, 20mM MgOAc and 0.2mM ATP (all from Sigma, St. Louis, MO, USA) was incubated for 1-60 minutes at 37°C with 0-20 casein units of the catalytic subunit of the cAMP dependent protein kinase (Promega, Madison, WI. USA) and 0-200$\mu$M cAMP dependent protein kinase inhibitor. Emission (excitation wavelength 395nm or 470nm) and excitation (emission wavelength 508nm) spectra were acquired for all samples using the LS50B Luminescence Spectrometer and the FL data Manager software package (Perkin Elmer). The spectra were subsequently compared by using the Graph Builder software package (Perkin Elmer).

As can be seen from Fig. 3, the fluorescence intensity of wild-type GFP increases approximately two-fold when incubated with the catalytic subunit of the cAMP dependent protein kinase. Moreover, 5$\mu$m cAMP dependent protein kinase inhibitor inhibits the effect of the catalytic subunit of the cAMP dependent protein kinase.

Figure 3 shows emission spectra of 0.5$\mu$g wild-type GFP (purified as described in Example 6) in 40mM Tris, pH 7.4, 20mM MgOAc and 0.2mM ATP (all from Sigma), incubated for 5 minutes at 37°C with 10 casein units of the catalytic subunit of the

cAMP dependent protein kinase (Promega) with or without 5$\mu$M cAMP depende protein kinase inhibitor (PKI). The control (w/o PKA) was incubated 5 minutes at 37° without the catalytic subunit of the cAMP dependent protein kinase. The excitatic wavelength was 395nm. RFI in the figure means relative fluorescence intensity.

## Example 7

Characterization of wild-type GFP as a PKA activity probe in mammalian cells.

The green fluorescent proteins exhibiting phosphorylation-induced changes fluorescence are investigated further by expression in mammalian cells.

Inserts of the respective plasmids are isolated by digestion with the restricti endonucleases HindIII and BamHI and ligated into the HindIII and BamHI sites of t MCS of the mammalian pZEO-SV vector (Invitrogen, San Diego, California, US Subsequently, Baby Hamster Kidney (BHK) are transfected with the resulting plasm constructs according to the standard calcium phosphate-DNA precipitate protocol ( Sambrook et al., op. cit. at 16.33-16.35). Stable transfectants with high expression of t recombinant probes are identified and cloned after 6-14 days in culture by quantifyi the fluorescence in an image analysis system, which consists of a Nikon Diaphot 2 microscope with a temperature controlled stage, a slow scan CCD camera (T.I.L. Photonics), a polychromatic light source (T.I.L.L. Photonics), and a PC based ima analysis software package (FUCAL from T.I.L.L. Photonics). Alternatively, t fluorescence properties are monitored in a photometer based system. In this system t CCD camera is replaced by a photomultiplier D104 (PTI, Canada).

The clones are cultured for a couple of days in glass coverslip chambers (NUN Copenhagen, Denmark) before image analysis.

The ability of the clones to detect changes in protein kinase A activity is characteri by elevating intracellular cAMP level by challenging the cells with forskolin (0.1-10 $\mu$ or dibutyryl-cAMP (1-100 $\mu$M) and monitoring the associated change of spect

properties. The stimulant-induced changes of fluorescence properties are monitored continuously using above mentioned imaging system. Combining imaging with photometry makes it possible to characterize the response of the recombinant probes in both high spatial and high temporal resolution.

# INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

**A.** The indications made below relate to the microorganism referred to in the description on page 6, line 12.

**B. IDENTIFICATION OF DEPOSIT** — Further deposits are identified on an additional sheet

Name of depositary institution

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

| Date of deposit | Accession Number |
|---|---|
| 21.09.1995 | DSM No. 10260 |

**C. ADDITIONAL INDICATIONS** *(leave blank if not applicable)* — This information is continued on an additional sheet

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

**D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated Stat*

**E. SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Access Number of Deposit")*

| For receiving Office use only | For International Bureau use only |
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Form PCT/RO/134 (July 1992)

## CLAIMS

1. A method of detecting a biologically active substance affecting intracellular processes, the method comprising

(a) culturing a cell containing a DNA sequence coding for

(i) a green fluorescent protein (GFP) having a protein kinase recognition site, or

(ii) a green fluorescent protein wherein one or more amino acids have been substituted, inserted or deleted to provide a binding domain of a second messenger or an enzyme recognition site, or

(iii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GFP and an enzyme recognition site or a binding domain for a second messenger

under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample suspected of containing a biologically active substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining any change in the fluorescence compared to the fluorescence measured in step (b), such change being indicative of the presence of a biologically active substance in said sample.

2. A method of detecting a biologically active substance affecting intracellular processes according to claim 1, the method comprising

(a) culturing a cell containing a DNA sequence coding for

(i) green fluorescent protein wherein one or more amino acids have be substituted, inserted or deleted to provide a binding domain of a second messeng or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GI and a binding domain of a second messenger or an enzyme recognition site

under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample suspected of containing a biologically act substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining a change in the fluorescence compared to the fluorescence measured in step (b), su change being indicative of the presence of a biologically active substance in said samp

3. A method according to any one of the preceding claims, wherein the cell is eukaryotic cell.

4. A method according to any one of the preceding claims, wherein the cell is a ye cell or a mammalian cell.

5. A method according to any one of the preceding claims, wherein the binding dom is a receptor.

6. A method according to any one of the preceding claims, wherein the binding dom is a cyclic AMP receptor or a part thereof capable of binding cyclic AMP.

7. A method according to any one of the preceding claims, wherein the enzy recognition site is a protein kinase recognition site.

8. A method according to any one of the preceding claims, wherein the protein kinase recognition site is selected from the group consisting of protein kinase A, protein kinase C, the insulin receptor, and the Src kinase.

9. A method according to any one of the preceding claims, wherein the gene encoding GFP is derived from *Aequorea victoria*.

10. A method of characterizing the biological activity of a substance with biological activity, the method comprising

(a) culturing a cell containing a DNA sequence coding for

(i) a green fluorescent protein having a protein kinase recognition site, or

(ii) a green fluorescent protein wherein one or more amino acids have been substituted, inserted or deleted to provide a binding domain of a second messenger or an enzyme recognition site, or

(iii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GFP and a binding domain of a second messenger or an enzyme recognition site

under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample of a biologically active substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining any change in the fluorescence compared to the fluorescence measured in step (b), said change being characteristic of the biological activity of the biologically active substance in said sample.

11. A method of characterizing the biological activity of a substance with biologi activity according to claim 10, the method comprising

(a) culturing a cell containing a DNA sequence coding for

(i) green fluorescent protein wherein one or more amino acids have be substituted, inserted or deleted to provide a binding domain of a second messeng or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified Gl and a binding domain of a second messenger or an enzyme recognition site

under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample of a biologically active substance affecti intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining a change in the fluorescence compared to the fluorescence measured in step (b), sa change being characteristic of the biological activity of the biologically active substan in said sample.

12. A method according to any one of claims 10 or 11, wherein the cell is a eukaryo cell.

13. A method according to any one of claim 10, 11 or 12, wherein the cell is a yeast c or a mammalian cell.

14. A method according to any one of claims 10, 11, 12 or 13, wherein the bindi domain is a receptor.

15. A method according to any one of claims 10, 11, 12, 13 or 14, wherein the binding domain is a cyclic AMP receptor or a part thereof capable of binding cyclic AMP.

16. A method according to any one of claims 10, 11, 12, 13, 14 or 15 wherein the enzyme recognition site is a protein kinase recognition site.

17. A method according to any one of claims 10, 11, 12, 13, 14, 15 or 16, wherein the protein kinase recognition site is selected from the group consisting of protein kinase A, protein kinase C, the insulin receptor, and the Src kinase.

18. A method according to any one of claims 10, 11, 12, 13, 14, 15, 16, or 17 wherein the gene encoding GFP is derived from *Aequorea victoria*.

19. A cell containing a DNA sequence coding for

(i) green fluorescent protein wherein one or more amino acids have been substituted, inserted or deleted to provide a binding domain of a second messenger or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified GFP and a binding domain of a second messenger or an enzyme recognition site,

and capable of expressing said DNA sequence.

20. A cell according to claim 19, which is a eukaryotic cell.

21. A cell according to any one of claims 19 or 20, which is yeast cell or a mammalian cell.

22. A cell according to any one of claims 19, 20 or 21 wherein the binding domain is a receptor.

23. A cell according to any one of claims 19, 20, 21 or 22, wherein the binding dom is a cyclic AMP receptor or a part thereof capable of binding cyclic AMP.

24. A cell according to any one of claims 19, 20, 21, 22 or 23 wherein the enzy recognition site is a protein kinase recognition site.

25. A cell according to any one of claims 19, 20, 21, 22, 23 or 24, wherein the pro kinase recognition site is selected from the group consisting of protein kinase A, pro kinase C, the insulin receptor, and the Src kinase.

26. A cell according to any one of claims 19, 20, 21, 23, 24 or 25 wherein the g encoding GFP is derived from *Aequorea victoria*.

27. A DNA construct comprising a DNA sequence coding for

(i) green fluorescent protein wherein one or more amino acids have b substituted, inserted or deleted to provide a binding domain of a second messer or an enzyme recognition site, or

(ii) a hybrid polypeptide of green fluorescent protein (GFP) or a modified C and a binding domain of a second messenger or an enzyme recognition site.

28. A DNA construct according to claim 27, wherein the binding domain is a recep

29. A cell according to any one of claims 27 or 28, wherein the binding domain cyclic AMP receptor or a part thereof capable of binding cyclic AMP.

30. A DNA construct according to any one of claims 27, 28 or 29 wherein the enz recognition site is a protein kinase recognition site.

31. A DNA construct according to any one of claims 27, 28, 29 or 30, wherein the protein kinase recognition site is selected from the group consisting of protein kinase A, protein kinase C, the insulin receptor, and the Src kinase.

32. A DNA construct according to any one of claims 27, 28, 29, 30 or 31 wherein the gene encoding GFP is derived from *Aequorea victoria*.

33. A method of detecting a biologically active substance affecting intracellular processes according to claim 1, the method comprising

(a) culturing a cell containing a DNA sequence coding for wild-type green fluorescent protein (GFP) having a protein kinase recognition site under conditions permitting expression of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample suspected of containing a biologically active substance affecting intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining any change in the fluorescence compared to the fluorescence measured in step (b), such change being indicative of the presence of a biologically active substance in said sample.

34. A method according to the preceding claim, wherein the cell is a eukaryotic cell.

35. A method according to any one of claims 33 or 34, wherein the cell is a yeast cell or a mammalian cell.

36. A method according to any one of claims 33, 34 or 35 wherein the protein kinase recognition site is selected from the group consisting of protein kinase A, protein kinase C, the insulin receptor, and the Src kinase.

37. A method according to any one of claims 33, 34, 35 or 36 wherein the pro kinase recognition site is a protein kinase A recognition site.

38. A method according to any one of claims 33, 34, 35, 36 or 37, wherein the D sequence encoding GFP is derived from *Aequorea victoria*.

39. A method according to any one of claims 33, 34, 35, 36, 37 or 38, wherein the D sequence is identical to the nucleotide sequence of Fig. 4a or any functional analo thereof.

40. A method of characterizing the biological activity of a substance with biolog activity according to claim 10, the method comprising

(a) culturing a cell containing a DNA sequence coding for a wild-type green fluores protein having a protein kinase recognition site under conditions permitting expres of the DNA sequence,

(b) measuring the fluorescence of the cell,

(c) incubating the cell with a sample of a biologically active substance affec intracellular processes, and

(d) measuring the fluorescence produced by the incubated cell and determining change in the fluorescence compared to the fluorescence measured in step (b), change being characteristic of the biological activity of the biologically active subst in said sample.

41. A method according to claim 40, wherein the cell is a eukaryotic cell.

42. A method according to any one of claims 40 or 41, wherein the cell is a yeast or a mammalian cell.

43. A method according to any one of claims 40, 41 or 42, wherein the protein kinase recognition site is a protein kinase A recognition site.

44. A method according to any one of claims 40, 41, 42 or 43, wherein the DNA sequence encoding GFP is derived from *Aequorea victoria*.

45. A method according to any one of claims 40, 41, 42, 43 or 44, wherein the DNA sequence is the nucleotide sequence of Fig. 4a or any functional analogue thereof.

46. A DNA construct containing the nucleotide sequence of Fig. 4a.

47. A transformation vector containing the nucleotide sequence of Fig. 4a.

48. A transformed cell containing the DNA construct of claim 46.

49. A transformed cell according to the preceding claim, characterised by being a mammalian cell.

50. The transformed E. coli having the deposition No. DSM 10260.

51. An *in vitro* assay for measuring protein kinase A activity, wherein purified wild-type GFP is added to a biological sample, preferably a cell extract, and any change in fluorescence of the GFP is recorded.

52. An *in vitro* assay for monitoring cAMP levels in a sample, wherein wild-type GFP and tetrameric PKA having two regulatory and two catalytic subunits are added to a sample, preferably a cell extract, and any change in fluorescence is recorded.

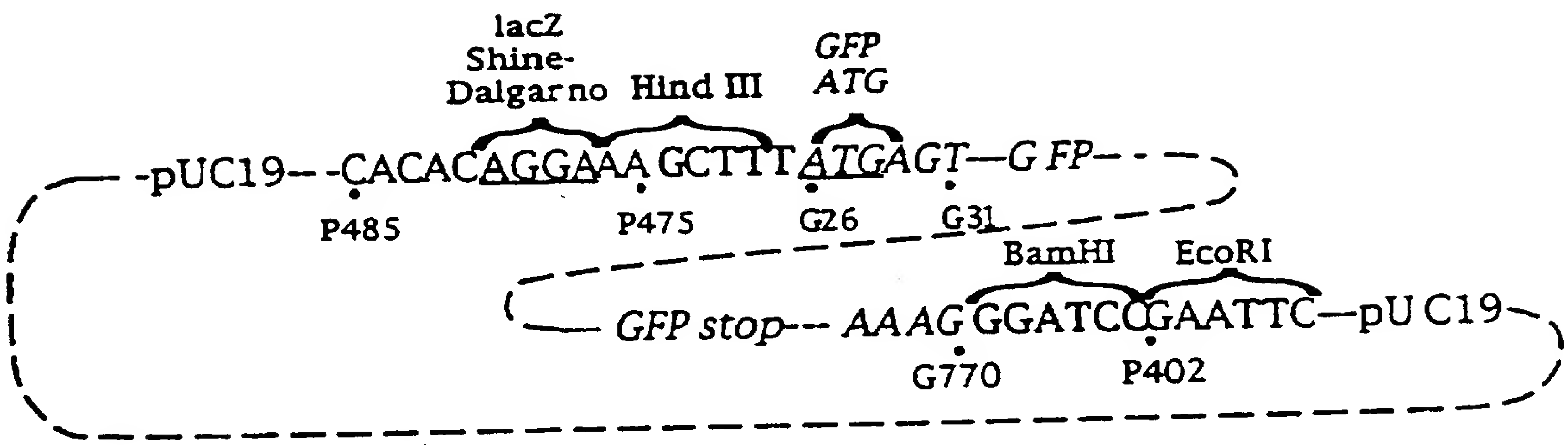

lacZ Shine-Dalgarno
Hind III
GFP ATG
-pUC19--CACACAGGAAAGCTTTATGAGT—GFP--
P485
P475
G26
G31
BamHI
EcoRI
GFP stop-- AAAGGGATCCGAATTC—pUC19
G770
P402

Fig. 1

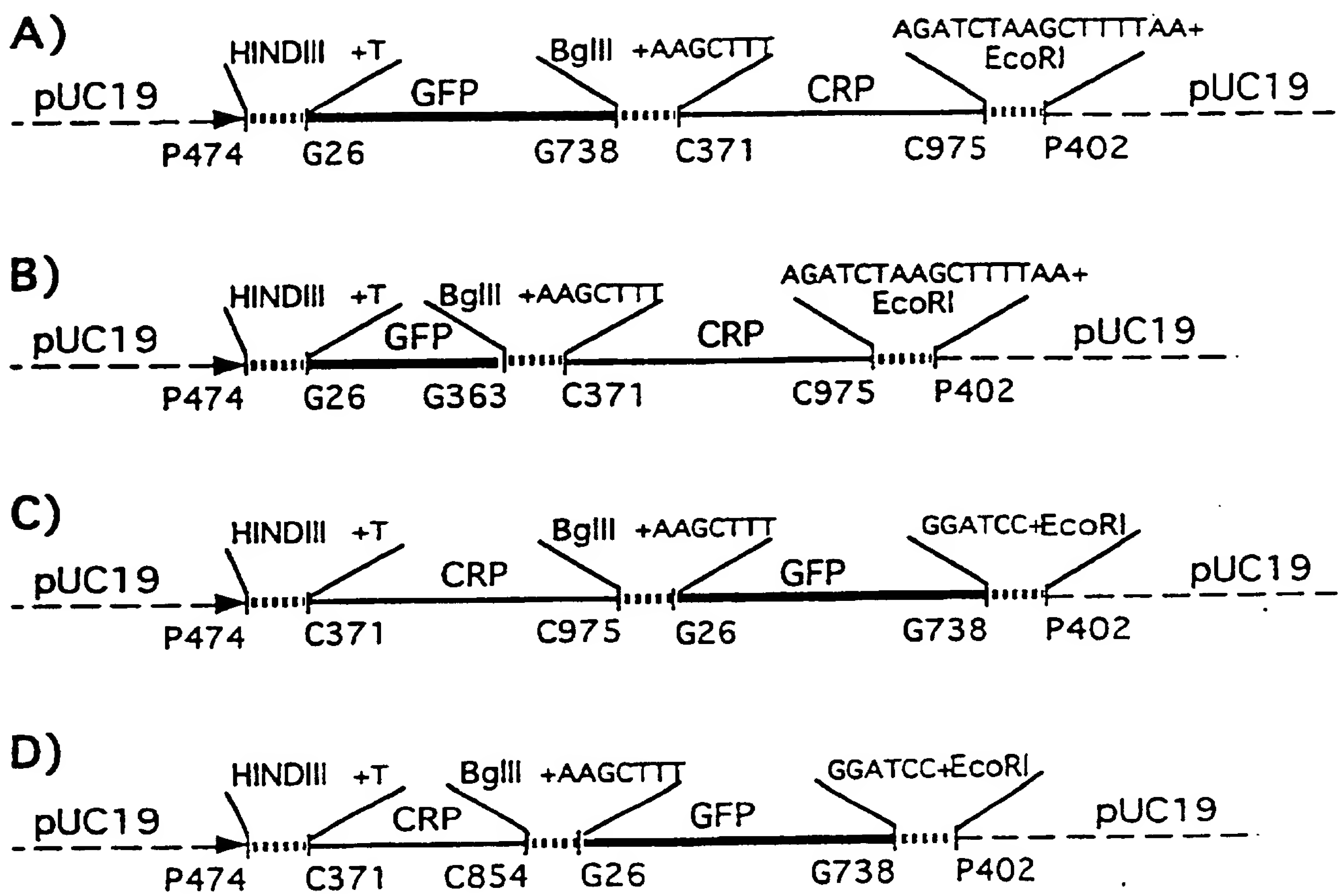
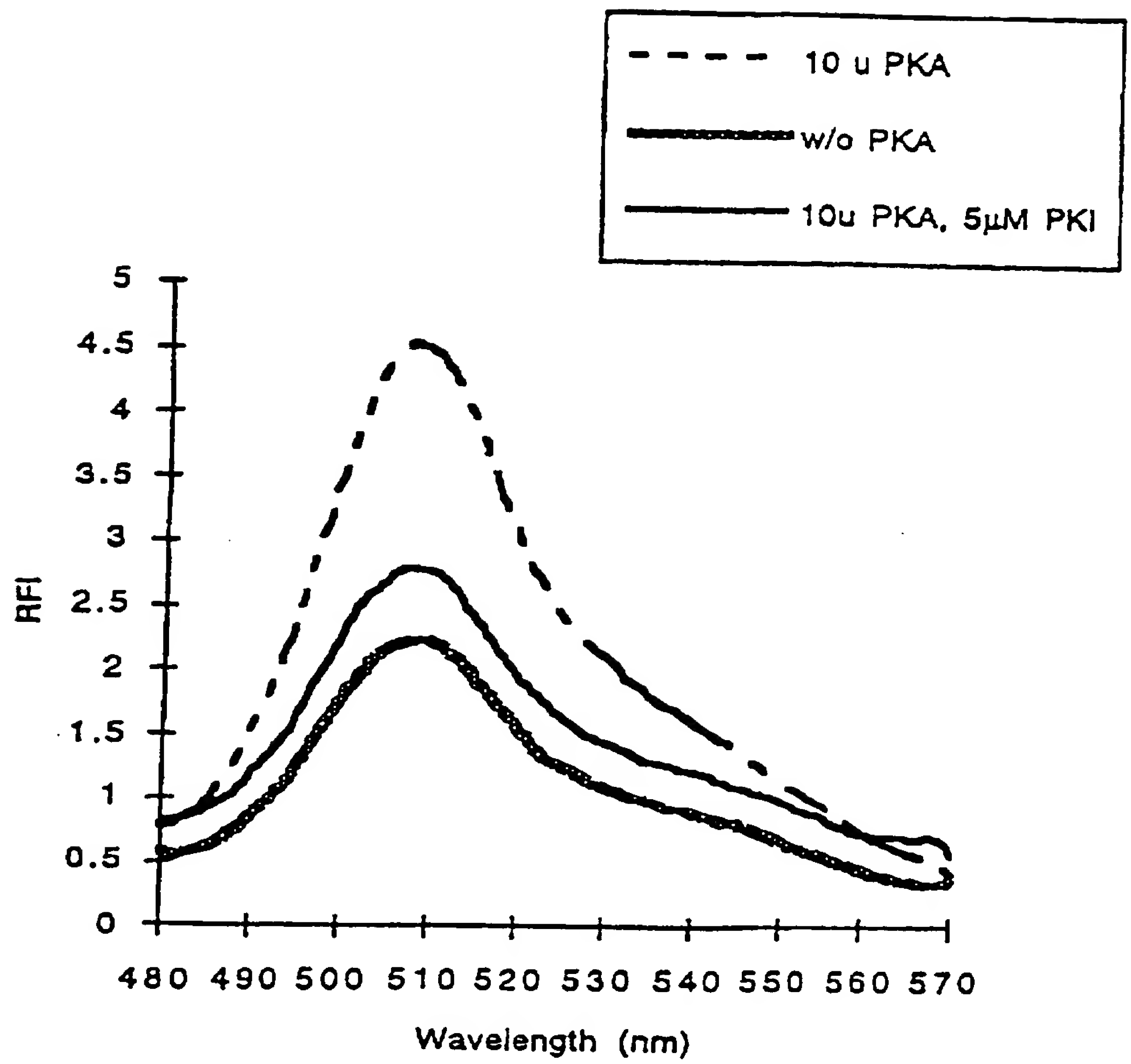

A)
pUC19
HINDIII +T
GFP
BglII +AAGCTTT
CRP
AGATCTAAGCTTTTTAA+
EcoRI
pUC19
P474
G26
G738
C371
C975
P402
B)
pUC19
HINDIII +T
BglII +AAGCTTT
GFP
CRP
AGATCTAAGCTTTTTAA+
EcoRI
pUC19
P474
G26
G363
C371
C975
P402
C)
pUC19
HINDIII +T
CRP
BglII +AAGCTTT
GFP
GGATCC+EcoRI
pUC19
P474
C371
C975
G26
G738
P402
D)
pUC19
HINDIII +T
BglII +AAGCTTT
CRP
GFP
GGATCC+EcoRI
pUC19
P474
C371
C854
G26
G738
P402

Fig. 2

Figure 3


10 u PKA
w/o PKA
10u PKA, 5μM PKI
RFI
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
480
490
500
510
520
530
540
550
560
570
Wavelength (nm)

Nucleotide sequence (764bp) of GFP
(Hind3-EcoRI fragment)

AAGCTTTATGAGTAAAGGAGAAGAACTTTTCACTGGAGTT
GTCCCAATTCTTGTTGAATTAGATGGCGATGTTAATGGGC
AAAAATTCTCTGTTAGTGGAGAGGGTGAAGGTGATGCAAC
ATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGG
AAGCTACCTGTTCCATGGCCAACGCTTGTCACTACTTTCT
CTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATAT
GAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGT
TATGTACAGGAAAGAACTATATTTTACAAAGATGACGGGA
ACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATAC
CCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAA
GAAGATGGAAACATTCTTGGACACAAAATGGAATACAACT
ATAACTCACATAATGTATACATCATGGCAGACAAACCAAA
GAATGGCATCAAAGTTAACTTCAAAATTAGACACAACATT
AAAGATGGAAGCGTTCAATTAGCAGACCATTATCAACAAA
ATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAA
CCATTACCTGTCCACGCAATCTGCCCTTTCCAAAGATCCC
AACGAAAAGAGAGATCACATGATCCTTCTTGAGTTTGTAA
CAGCTGCTGGGATTACACATGGCATGGATGAACTATACAA
ATAAATGTCCAGACTTCCAATTGACACTAAAGGGATCCGA
ATTC

Fig. 4a

Amino acid sequence:

Start codon ATG corresponds to position 8 in the nucleotide sequence above and stop codon TAA corresponds to position 722.

```
1/1                                         31/11
atg agt aaa gga gaa gaa ctt ttc act gga gtt gtc cca att ctt gtt gaa tta gat ggc
Met ser lys gly glu glu leu phe thr gly val val pro ile leu val glu leu asp gly

61/21                                       91/31
gat gtt aat ggg caa aaa ttc tct gtt agt gga gag ggt gaa ggt gat gca aca tac gga
asp val asn gly gln lys phe ser val ser gly glu gly glu gly asp ala thr tyr gly

181/61                                      211/71
aaa ctt acc ctt aaa ttt att tgc act act ggg aag cta cct gtt cca tgg cca acg ctt
lys leu thr leu lys phe ile cys thr thr gly lys leu pro val pro trp pro thr leu

121/41                                      151/51
gtc act act ttc tct tat ggt gtt caa tgc ttt tca aga tac cca gat cat atg aaa cag
val thr thr phe ser tyr gly val gln cys phe ser arg tyr pro asp his met lys gln

241/81                                      271/91
cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa aga act ata ttt tac
his asp phe phe lys ser ala met pro glu gly tyr val gln glu arg thr ile phe tyr

301/101                                     331/111
aaa gat gac ggg aac tac aag aca cgt gct gaa gtc aag ttt gaa ggt gat acc ctt gtt
lys asp asp gly asn tyr lys thr arg ala glu val lys phe glu gly asp thr leu val

361/121                                     391/131
aat aga atc gag tta aaa ggt att gat ttt aaa gaa gat gga aac att ctt gga cac aaa
asn arg ile glu leu lys gly ile asp phe lys glu asp gly asn ile leu gly his lys

421/141                                     451/151
atg gaa tac aac tat aac tca cat aat gta tac atc atg gca gac aaa cca aag aat ggc
met glu tyr asn tyr asn ser his asn val tyr ile met ala asp lys pro lys asn gly

481/161                                     511/171
atc aaa gtt aac ttc aaa att aga cac aac att aaa gat gga agc gtt caa tta gca gac
ile lys val asn phe lys ile arg his asn ile lys asp gly ser val gln leu ala asp

541/181                                     571/191
cat tat caa caa aat act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac
his tyr gln gln asn thr pro ile gly asp gly pro val leu leu pro asp asn his tyr

601/201                                     631/211
ctg tcc acg caa tct gcc ctt tcc aaa gat ccc aac gaa aag aga gat cac atg atc ctt
leu ser thr gln ser ala leu ser lys asp pro asn glu lys arg asp his met ile leu

661/221                                     691/231
ctt gag ttt gta aca gct gct ggg att aca cat ggc atg gat gaa cta tac aaa taa
leu glu phe val thr ala ala gly ile thr his gly met asp glu leu tyr lys OCH
```

Fig. 4b

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00052

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC6: C12Q 1/00, C07K 14/435
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, CA, USPATFULL, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| P,X | WO 9521191 A1 (WARD, WILLIAM ET AL), 10 August 1995 (10.08.95), see page 17<br>-- | 1-52 |
| P,X | WO 9507463 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK), 16 March 1995 (16.03.95)<br>-- | 1-52 |
| A | US 4220450 A (MAGGIO), 2 Sept 1980 (02.09.80)<br>-- | 1-52 |
| A | SCIENCE, Volume 263, February 1994, MARTIN CHALFIE ET AL, "GREEN FLUORESCENT PROTEIN AS A MARKER FOR GENE EXPRESSION" page 802<br>--<br>-------- | 1-52 |

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" ertier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or prio date and not in conflict with the application but cited to understar the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combinat being obvious to a person skilled in the art
- "&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 3 May 1996 | 07 -05- 1996 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br><br>Carolina Gomez<br>Telephone No. +46 8 782 25 00 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.
PCT/DK 96/00052

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| WO-A1- | 9521191 | 10/08/95 | NONE | | |
| WO-A1- | 9507463 | 16/03/95 | NONE | | |
| US-A- | 4220450 | 02/09/80 | DE-A- | 2913549 | 18/10/79 |
| | | | FR-A,B- | 2422165 | 02/11/79 |
| | | | GB-A,B- | 2018424 | 17/10/79 |
| | | | JP-C- | 1491117 | 07/04/89 |
| | | | JP-A- | 54151894 | 29/11/79 |
| | | | JP-B- | 63037347 | 25/07/88 |
| | | | US-A- | 4277437 | 07/07/81 |